Identification and characterization of differentially expressed genes during incompatible interaction between the foliar rust *Melampsora larici-populina* and poplar

Z.J. Chen, Z.M. Cao and Z.D. Yu

College of Forestry, Northwest A&F University, Yangling, Shaanxi, China

Corresponding author: Z.M. Cao
E-mail: zmcao@nwsuaf.edu.cn

ABSTRACT. Poplars are extensively cultivated worldwide, and their susceptibility to the foliar rust fungus leads to considerable damages in plantations. To better understand the molecular basis of poplar responses to the foliar rust, suppression subtractive hybridization was used to identify the potential important or novel genes involved in the *Populus szechuanica* infection by *Melampsora larici-populina*. A total of 515 expressed sequence tags (ESTs) with high quality were obtained and clustered into 66 contigs and 75 singletons to give a set of 141 uniESTs. The nucleotide Basic Local Alignment Search Tool (BLASTn) program was used to search for homologous sequences of the uniESTs in the GenBank database. Among them, 92.9% showed homology to the poplar genome, and 2% showed similarity to the rust fungus genome. In addition, homology to known genes was analyzed by the BLASTx algorithm, and approximately 50% of the uniESTs were significantly homologous to genes encoding proteins with known functions. Based on a reverse transcription-polymerase chain reaction (PCR) and quantitative PCR approach, five uniESTs

were analyzed, and the results showed that the expression level of the thaumatin-like gene was highest at 72 h post-inoculation, and the pathogenesis-related protein 1 gene was highest at 48 h post-inoculation. The information generated in this study provides new clues to aid in the understanding of incompatibility between poplar and the foliar rust.

Key words: Expressed sequence tags; Defense gene; *Melampsora larici-populina*; *Populus szechuanica*; Suppression subtractive hybridization

INTRODUCTION

Poplar plays an important role not only in economics as a naturally fast growing tree for wood production and in plant science as a model plant for perennial species but also in ecology as a sustainable bioenergy crop with a high carbon storage capacity (Rubin, 2008). Currently, *Melampsora larici-populina* represents the major threat of poplars in plantations worldwide, especially in Europe, North America, and China (Pinon and Frey, 2005; Cao et al., 2012). This biotropic fungus caused the most important foliar disease of poplar that greatly reduced the photosynthetic capacity, biomass production, and overall economic value of poplar trees in poplar plantation (Major et al., 2010; Covarelli et al., 2013; Wan et al., 2013). Breeding some resistance cultivars was the traditional method to reduce losses of this disease, but the long generation time of poplars made it impossible to match the evolutionary rates of the pathogen, which goes through several generations every year (Duplessis et al., 2009; Hacquard et al., 2011). Therefore, novel strategies to control this disease are needed, which essentially rely on a better understanding of the molecular mechanisms of the host and pathogen interaction.

Poplar-rust interaction is the emerging model of plant-pathogen interactions, especially in perennial plant species. Recently, the black cottonwood *Populus trichocarpa* "Nisqually-1" (http://genome.jgi.doe.gov/pages/blast.jsf?db=Poptr1_1) and *M. larici-populina* (http://genomeportal.jgi-psf.org/pages/blast.jsf?db=Mellp1) have been sequenced by the Joint Genome Institute (JGI, Department of Energy, USA), and the analysis of these genomes is a great opportunity to identify fungal effector genes and poplar signaling genes during *Populus-Melampsora* interactions (Duplessis et al., 2009, 2011). To date, approximately 400 nucleotide-binding site leucine-rich repeat genes, representing a large class of plant resistance genes responsible for pathogen effector recognition and leading to complete resistance through effector-triggered immunity in many pathosystems (Dodds and Rathjen, 2010), were identified in the poplar genome by genome-wide analysis. This was twice the number reported in *Arabidopsis thaliana* (402 versus 178, respectively) (Hacquard et al., 2011). In addition to the large number of resistance genes, an expanded gene family encoding thaumatin-like proteins (TLPs), corresponding to the pathogenesis-related (PR-5) proteins, illustrates an overrepresentation of defense-related genes in poplar with 42 genes identified in *P. trichocarpa* (Petre et al., 2011). In several plant-pathogen systems, a systemic response also occurs throughout the whole plant, but this phenomenon has not yet been clearly documented in poplar; these responses include Ca^{2+} influxes, activation of mitogen-activated protein kinase cascades, and generation of reactive oxygen

species (Menges et al., 2008). Additionally, the transcriptome profilings of poplar-rust interactions in North America (Azaiez et al., 2009; Petre et al., 2012) and in Europe (Rinaldi et al., 2007) have been reported.

        In order to better understand the mechanisms associated with the poplar genes potentially related to poplar defense response during the infection course of the foliar rust fungus *M. larici-populina* in China, which is critical to better control and management of this disease, we used *Populus szechuanica* as a tested host, a wild poplar species in China, which is resistant to most isolates of *M. larici-populina*, and has been widely used as differentiating host of the foliar rust races in the recent years (Liu et al., 2008; Chen et al., 2010; Cao et al., 2012). Therefore, we first constructed a suppression subtractive hybridization (SSH) library of the incompatible interaction between *P. szechuanica* and *M. larici-populina*, and then the dynamic expression of some rust-induced resistance genes were further confirmed by reverse transcription-polymerase chain reaction (RT-PCR) and quantitative PCR (qPCR).

MATERIAL AND METHODS

Plant materials and inoculation procedures

        The avirulent isolate Sb_{052} of *M. larici-populina* (MLP) used in this study was from the College of Forestry at Northwest A&F University, and all experiments were performed on 2-3-year-old cutting plants of *P. szechuanica*, which had been potted and grown in a glasshouse for 12 weeks. The urediniospores of the MLP isolate Sb_{052} were multiplied on *P. purdomii* plant leaves, which are susceptible to most of Chinese MLP isolates, and collected from 12 to 20 days post-inoculation. The spores were dried and kept in a vacuum container at 4°C. Fully expanded leaves from leaf plastochron index 5 to 8 were washed by clean tap water and spray inoculated on their abaxial surface with urediospore suspension (1-2 mg/mL) or with tap water as a control (mock-inoculated leaves). To facilitate pathogen infection, post-inoculated and mock-inoculated plants were placed in a growth chamber with high humidity at 25-27°C for 24 h. Materials were harvested at different time points, including 6, 12, 24, 48, 72, 96, and 120 h post-inoculation (hpi), and the leaves were immediately snap-frozen in liquid nitrogen until RNA extraction.

RNA extraction and SSH

        Total RNA was isolated from *P. szechuanica* Schneid. leaves harvested at different time points using the Trizol reagent (Invitrogen, Carlsbad, CA, USA), and mRNA was purified with the Oligotex mRNA Spin-Column Kit (Qiagen, Hilden, Germany) according to the manufacturer protocol. Briefly, tester RNA was prepared by pooling equal amounts of RNA from infection leaves harvested at 12, 24, 48, and 72 hpi, while driver RNA was pooled from the control leaves. The cDNA was synthesized using the SMART PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA, USA), and SSH was carried out using the PCR-Select™ cDNA Subtraction Kit (Clontech) according to manufacturer instructions, with slight modifications. Driver and tester cDNAs were digested with the restriction enzyme *Rsa*I and purified with the QIAquick PCR Purification Kit (Qiagen). The tester cDNA was subdivided into two pools and each was ligated with a different cDNA adaptor (supplied in kit). Finally, two

rounds of hybridization and PCR amplification were carried out to normalize and enrich the differentially expressed sequence tags (ESTs) according to the manufacturer protocol. The subtraction efficiency was validated by PCR analysis using internal control β-actin primers (forward: CGTTTGGATCTTGCTGGTC, reverse: ACTTCTGGGCAACGGAATC). The subtracted PCR products were then inserted into the pGEMT Easy vector (Promega, Madison, WI, USA) and transformed into *Escherichia coli* Top10 competent cells, which were plated on solid Luria-Bertani (LB) supplemented with 50 mg/L ampicillin. The bacterial colonies were hand-picked from the plates and transferred to 96-well microtiter plates containing LB broth supplemented with 50 mg/L ampicillin and grown overnight at 37°C. The colony PCR was performed to amplify the inserted ESTs by T7 and SP6 primers, and the positive clones were selected for sequence analysis by the DNA sequencer (Applied Biosystems 3730 Genetic Analyzer, USA).

EST analysis

The sequences were first screened against the UniVec database (http://www.ncbi.nlm.nih.gov/Vec-Screen/UniVec.html) and the *E. coli* genome sequence to remove vector, adaptor, and *E. coli* genome contamination. The resulting high-quality sequences were then clustered and assembled using the CAP3 program (Huang and Madan, 1999). In total, the unigenes were obtained and analyzed using nucleotide Basic Local Alignment Search Tool (BLASTn) with *P. trichocarpa* v1.1 (http://genome.jgi-psf.org/pages/blast.jsf?db=Poptr1_1) and *M. larici-populina* v1.0 (http://genome.jgi-psf.org/pages/blast.jsf?db=Mellp1) from the JGI. The gene ontology (GO) annotations for the sequences were derived using their Uniprot annotation. The Uniprot database was used because it had extensive GO mapping. The GO annotation for level 2 was extracted for each library and used for further analysis (http://www.blast2go.com/b2ghome).

RT-PCR and qPCR

Total leaf RNA was isolated by Trizol reagent (Invitrogen) from two tested populations. First-strand cDNA was synthesized with RT primer mixture (Oligo dT primer and Random 6 mers) using PrimeScript® RT Reagent Kit with gDNA Eraser (TaKaRa, Dalian, China) according to manufacturer instructions. The *Populus* ubiquitin gene was used as an endogenous control, and all primers for the candidate genes and *Populus* ubiquitin were designed by the Primer v5.0 software (PREMIER Biosoft International, Palo Alto, CA, USA), which were shown in Table 1. RT-PCR was conducted with the following program: an initial denaturation at 94°C for 5 min; 25-30 cycles of 94°C for 30 s, X°C (see Tm in Table 1) for 30 s, and 72°C for 1 min; and a final extension at 72°C for 5 min. The PCR products were detected on 2% agarose gels in 1X Tris, borate, ethylenediaminetetraacetic acid with ethidium bromide. qPCR was performed using a Bio-Rad CFX 96™ Real-Time Detection System and SYBR Green PCR Master Mix (TaKaRa) in a 20 μL reaction. All reactions were carried out in triplicate. The PCR mix included 100 ng cDNA for each candidate gene, 0.4 μM forward and reverse primers, and 10 μL 2X SYBR Green PCR Master Mix. The cycle conditions were as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 10 s, X°C (see Tm in Table 1) for 10 s, and 68°C for 20 s. The threshold cycle (Ct) was defined as the

cycle number at which the fluorescence intensity passed a predetermined threshold. The quantification of each candidate gene relative to the ubiquitin gene was calculated using the equation: $N = 2^{-\Delta\Delta Ct}$.

Table 1. Specific primers designed for RT-PCR and qPCR amplification of *Populus* transcripts.

Gene name	Sequence No.	Primer sequences (5'-3') (forward/reverse)	Size (bp)	Tm (°C)
PR-5	XM_002325041.1	CGAAACCAATGCCCTTACAC CAATCACCTGTCTCGCACAT	179	58
MIPS	XM_002310544.1	TGTCATCAAAGGCACCAAGA AATACAAGCCAGAGCAAACAAG	229	55
PR-1	XM_002333719.1 XM_002300258.1	CATGTGTTGGTGGAGAATGC TCGCGGAAATACATCGTTTC	253	58
β-1,3-glucanase	XM_002336667.1 XM_002299755.1	CATTGCAAACAAGTATGTCT AGCATCCGCCAATTTACAAG	499	55
PAL	EU603320 CT029896	CTCATCTAGGTGGCTTCCTT ATTTAGAGCCTCGCAACTTC	305	55
Ubiquitin	XM_002321377.1	GCAGGGAAACAGTGAGGAAGG TGGACTCACGAGGACAG	151	58

PR-5 = thaumatin-like protein gene; MIPS = myo-inositol 1-phosphate synthase gene; PR-1 = pathogenesis-related protein 1 gene; β-1,3-glucanase = beta-1,3-glucanase; PAL = phenylalanine ammonia lyase; Ubiquitin = endogenous control.

RESULTS AND DISCUSSION

SSH libraries of incompatible *populus-M. larici-populina* interaction

To isolate genes that were differentially expressed in the post-inoculation poplar, the SSH library was constructed using pooled mRNAs from poplar leaves that were infected by MLP and harvested at 12, 24, 48, and 72 hpi, and pooled mRNA samples from untreated leaves were used as drivers. The upregulated EST fragments were then cloned into the pGEM-T Easy vector, and a total of 550 clones from 600 randomly selected clones in the SSH library were confirmed to be recombinant by PCR with nested primers (supplied in kit). The recombinant plasmids of the 550 clones were isolated and sequenced. All sequences were retrieved in FASTA format with the BioXM software. Prior to assembly, sequencing reads were trimmed to remove vector and ribosomal sequences, poly(A/T) tails, and low-quality sequences, reducing the number of ESTs to 515. After trimming, the CAP3 program was used to assemble sequences at 98% sequence identity using a minimal overlap of 75 bp. The assembly resulted in 515 sequences formed by 66 contigs and 75 singlets. The longest uniEST was 1114 bp, and most of them were around 200 to 500 bp. The length of the singlets varied from 81 to 646 bp with an average of 286 bp. The maximum singlets were grouped under 200-300 bp in length. The contig length ranged from 135 to 1114 bp with an average of 392 bp, and 22.7% of the contigs extended from 300 to 400 bp. The number of ESTs grouped in each contig varied between 1 and 41, with 84.8% of the contigs containing 2-10 EST members. The two most abundant uniESTs observed in the library included an EST that is most similar to plastidic aldolase from *Ricinus communis* (34 copies) and an EST similar to 1-aminocyclopropane-1-carboxylate oxidase from *P. trichocarpa* (41 cop-

ies). Additionally, it has been reported that the G + C content of a uniEST can be useful to distinguish plant-derived sequences from fungal sequences in mixed cDNA populations (Qutob et al., 2000). Our data showed that the average G + C content of the poplar ESTs was 44.74%, which is similar to that of soybean, rice, and *Arabidopsis* ESTs, but less than that reported in the wheat 3B chromosome exon-coding sequence (Arabidopsis Genome Initiative, 2000; Qutob et al., 2000; Choulet et al., 2010).
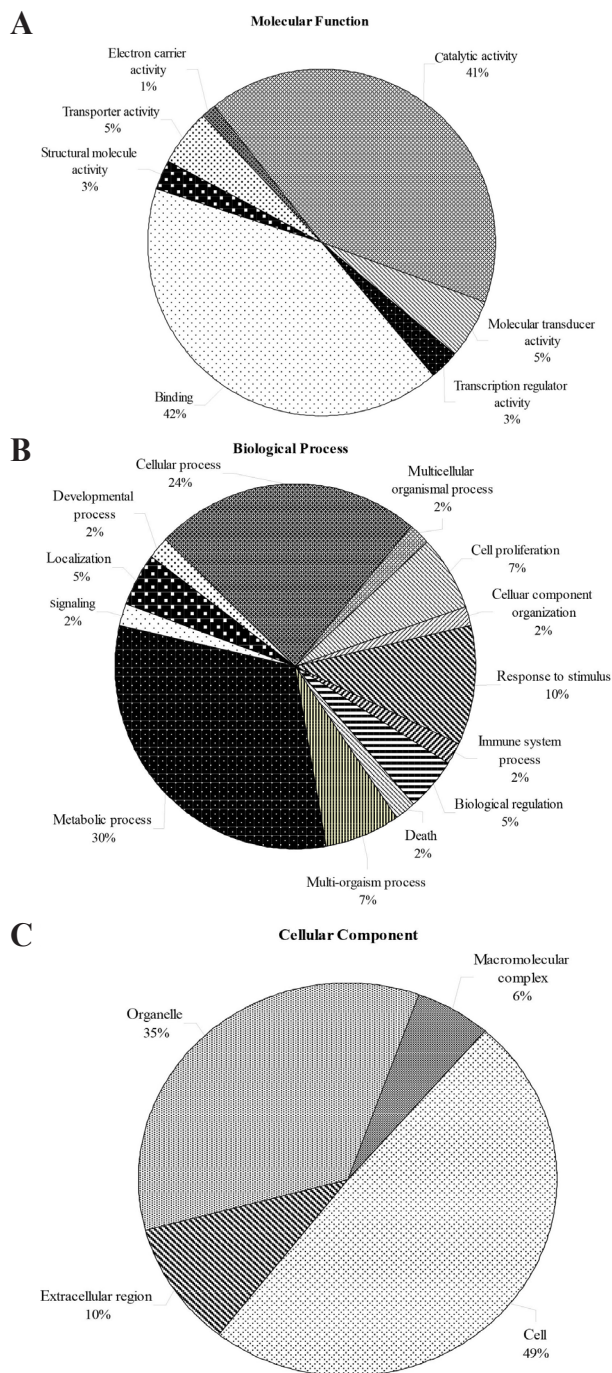
Functional annotation and disease-resistant expression profiling

Each of our uniESTs was compared to the *Populus* and *M. larici-populina* genome sequence through BLASTn in the JGI. Among them, 92.9% (131 of 141 unique sequences) showed homology to the poplar genome, and 2% (3 of 141 unique sequences) showed similarity to the rust fungus genome (**Table S1**). Furthermore, only contig 62 in the 10 uniESTs with no homology to the *Populus* genome exhibited homology (BLAST score ≥80) to the *Arabidopsis* ESTs; the remaining small fractions (9 ESTs) consisted of those with a weak score (<80) or no sequence similarity to any plant ESTs in the National Center for Biotechnology Information (NCBI) database. In addition, homology to known genes was analyzed by the BLASTx algorithm against the NCBI nonredundant protein database (E ≤ e-5 cutoff) in the Blast2GO software (http://www.blast2go.com/b2ghome) (Conesa et al., 2005). Unknown unique sequences (no BLAST identification or "hit" returned) totaled 80 (56.74%) of the 141 uniESTs (Table 2). We also analyzed the GO terms of 61 identified unigenes, which were organized into three categories representing molecular functions, biological processes, and cellular components (Gene Ontology Consortium, 2008). The sum of the poplar uniESTs per category did not add up to 100% because some sequences were classified into more than one category. Of the total unigene set, 51 (83.61%) were annotated into the molecular function category (describing the biochemical activity performed by the gene product), 50 (81.97%) into the biological process GO category (describing the ordered assembly of more than one molecular function), and 36 (59.02%) into the cellular component GO category (describing subcellular compartments of a cell). Among the molecular functions, the most highly represented categories were binding (42%) and catalytic activity (41%) (Figure 1A). Among the biological processes, the largest proportion of functionally assigned unigenes fell into metabolic process, cellular process, response to stimulus, multi-organism process, and cell proliferation, while signaling, localization, developmental process, multicellular organismal process, cellular component organization, immune system process, biological regulation, and death comprised 22% of the sequences (Figure 1B). For the cell component category, almost all unigene sequences were annotated into the cell-cell subcategory; 10% were categorized into extracellular region, and 7% were grouped into the macromolecular complex category (Figure 1C).

Transcripts were sampled from an immune-stimulated poplar leaf subtracted cDNA library by the EST approach in order to improve our studies of the *P. szechuanica* immune response to the entomopathogen MLP. From the ESTs involved in the poplar-MLP incompatible interaction, we identified several unigenes that were homologous to sequences of other species in this study (Table 2). Specific categories of defense response genes are summarized below. PR proteins are not present in healthy tissues, but they have been shown to accumulate after infection by pathogens, and the role of PR proteins was discussed in recent years (Sels et al., 2008). Compared with *Arabidopsis*, poplar has an abundance of defense-related genes,

Table 2. Expressed sequence tag similarity analysis.

Seq. name	Size (bp)	Description	E-Value	Identity	Copy
Contig1	604	DNA repair protein Sae2 [*Cordyceps militaris*]	1.6E-54	65.22%	2
Contig2	329	NBS-type disease resistance protein [*Populus trichocarpa*]	1.3E-6	57.00%	3
Contig7	231	Ribulose bisphosphate carboxylase oxygenase activase chloroplast [*Ricinus communis*]	8.1E-36	90.05%	5
Contig8	1087	Tropine dehydrogenase [*Ricinus communis*]	4.0E-120	83.40%	11
Contig10	392	Protein [*Populus trichocarpa*]	5.4E-8	47.80%	4
Contig11	745	Granulebound starch synthase [*Solanum tuberosum*]	2.5E-61	93.00%	14
Contig12	1114	Predicted protein [*Populus trichocarpa*]	2.1E-29	76.88%	15
Contig13	408	Protein [*Populus trichocarpa*]	9.2E-21	51.25%	4
Contig14	446	Galactinol synthase [*Populus trichocarpa* x *Populus deltoides*]	1.3E-74	95.45%	2
Contig15	229	NBS-type disease resistance protein [*Populus trichocarpa*]	1.1E-6	57.00%	15
Contig16	543	Hypothetical chloroplast RF2 [*Ruptiliocarpon caracolito*]	2.2E-38	92.10%	9
Contig23	489	RRNA intron-encoded homing endonuclease [*Medicago truncatula*]	7.5E-7	69.75%	3
Contig25	821	Predicted protein [*Populus trichocarpa*]	9.0E-6	32.00%	6
Contig37	480	Protein [*Populus trichocarpa*]	1.6E-28	50.65%	7
Contig38	342	Protein [*Populus trichocarpa*]	2.3E-8	47.95%	7
Contig39	409	Protein [*Populus trichocarpa*]	1.3E-29	53.80%	2
Contig41	498	PSI P700 apoprotein A2 [*Populus trichocarpa*]	6.2E-95	99.45%	10
Contig42	290	Protein [*Populus trichocarpa*]	3.3E-18	56.35%	2
Contig43	415	Ycf2 [*Cynomorium songaricum*]	2.1E-87	98.20%	2
Contig44	266	*Pinus taeda* anonymous locus cl1838contig1_02 genomic sequence [*Populus trichocarpa*]	1.2E-18	95.15%	5
Contig45	345	Protein [*Populus trichocarpa*]	2.5E-37	81.70%	14
Contig46	718	1-Aminocyclopropane-1-carboxylate oxidase [*Populus trichocarpa*]	9.3E-82	97.60%	41
Contig47	304	Phenylalanine ammonia lyase [*Populus trichocarpa*]	2.2E-17	82.38%	10
Contig48	245	Myo-inositol 1-phosphate synthase [*Solanum tuberosum*]	7.2E-43	95.90%	8
Contig49	455	Thaumatin-like protein [*Nicotiana tabacum*]	3.6E-94	80.65%	9
Contig50	499	Beta-1,3-glucanase [*Populus tremula* x *Populus tremuloides*]	5.8E-100	84.00%	12
Contig51	455	Pathogenesis-related protein 1 [*Populus tomentosa*]	9.2E-77	80.55%	2
Contig52	456	Pathogenesis-related protein 1 [*Populus tomentosa*]	2.5E-74	82.70%	2
Contig53	362	Protein [*Populus trichocarpa*]	5.3E-49	70.75%	2
Contig54	350	Multidrug resistance protein ABC transporter family [*Populus trichocarpa*]	1.6E-64	85.70%	2
Contig55	206	Metallothionein-like protein [*Populus trichocarpa* x *Populus deltoides*]	2.4E-31	83.75%	3
Contig56	261	Ribulose bisphosphate carboxylase oxygenase activase chloroplastic-like [*Ricinus communis*]	2.4E-28	87.35%	12
Contig58	337	Plastidic aldolase [*Ricinus communis*]	3.6E-64	98.85%	34
Contig59	221	Chloroplast photosystem I protein [*Populus tremula*]	2.7E-42	99.90%	10
Contig61	344	Rubisco activase [*Pinus halepensis*]	2.0E-54	98.60%	7
Contig63	353	Calvin cycle protein CP12-like [*Vitis vinifera*]	1.9E-58	77.95%	5
Contig64	466	DNA damage-inducible protein 1 [*Ricinus communis*]	3.8E-12	86.21%	4
Contig65	485	Protein [*Populus trichocarpa*]	3.8E-37	81.75%	4
Contig66	774	6-Phosphogluconate dehydrogenase [*Vitis vinifera*]	9.9E-94	93.30%	3
Singlet12	435	Hypothetical chloroplast RF2 [*Berberidopsis corallina*]	9.6E-73	97.35%	1
Singlet14	263	Protein [*Populus trichocarpa*]	2.5E-52	84.65%	1
Singlet17	517	Protein [*Populus trichocarpa*]	1.7E-27	50.80%	1
Singlet22	376	Protein [*Populus trichocarpa*]	1.9E-29	59.90%	1
Singlet23	485	Thaumatin-like protein [*Nicotiana tabacum*]	5.7E-101	79.05%	1
Singlet24	311	Trotein [*Populus trichocarpa*]	7.9E-29	64.33%	1
Singlet25	640	Beta-1,3-glucanase[*Populus tremula* x *Populus tremuloides*]	5.8E-129	84.45%	1
Singlet26	355	Glycolate oxidase [*Mangifera indica*]	1.2E-47	98.25%	1
Singlet27	289	Granulebound starch synthase[*Solanum tuberosum*]	4.53E-61	92.90%	1
Singlet28	400	Photosystem II protein N [*Oryza meridionalis*]	4.1E-23	94.15%	1
Singlet40	220	ATPase alpha subunit [*Arabidopsis thaliana*]	2.9E-16	96.75%	1
Singlet47	566	DNA repair protein SAE2 [*Cordyceps militaris* CM01]	9.9E-55	65.22%	1
Singlet63	384	Protein [*Populus trichocarpa*]	4.7E-15	46.95%	1
Singlet65	163	Xylem serine proteinase 1 [*Ricinus communis*]	1.3E-27	85.15%	1
Singlet66	459	Protein [*Populus trichocarpa*]	1.5E-73	80.70%	1
Singlet67	614	Galactinol synthase [*Populus trichocarpa* x *Populus deltoides*]	3.1E-109	92.05%	1
Singlet70	467	1-Aminocyclopropane-1-carboxylate oxidase [*Populus* x *canadensis*]	6.1E-49	78.65%	1
Singlet71	403	*Pinus taeda* anonymous locus cl1838contig1_02 genomic sequence	5.3E-18	94.95%	1
Singlet72	482	Protein [*Populus trichocarpa*]	3.7E-37	81.75%	1
Singlet73	647	Nucleotide-sugar transporter family protein [*Arabidopsis thaliana*]	2.6E-61	88.75%	1
Singlet74	548	Acyl:CoA ligase [*Populus trichocarpa*]	4.0E-12	90.67%	1
Singlet75	357	DEAD-box ATP-dependent RNA helicase [*Glycine max*]	3.8E-42	97.25%	1

A **Molecular Function**

Electron carrier
activity
1%

Transporter activity
5%

Structural molecule
activity
3%

Catalytic activity
41%

Molecular transducer
activity
5%

Transcription regulator
activity
3%

Binding
42%

B **Biological Process**

Cellular process
24%

Developmental
process
2%

Localization
5%

Signaling
2%

Multicellular
organismal process
2%

Cell proliferation
7%

Celluar component
organization
2%

Response to stimulus
10%

Immune system
process
2%

Biological regulation
5%

Death
2%

Multi-orgaism process
7%

Metabolic process
30%

C **Cellular Component**

Macromolecular
complex
6%

Organelle
35%

Extracellular region
10%

Cell
49%

Figure 1. Gene ontology annotations and functional analyses of the unigenes with the automated Blast2GO software. **A.** Molecular function. **B.** Biological process. **C.** Cellular component. The percentage of gene transcripts in each group is listed.

including thaumatin-like (PR-5), β-1,3-glucanases (PR-2), and pathogenesis-related protein 1 (PR-1), which likely represent poplar PR families (Tuskan et al., 2006). Consistent with *Arabidopsis* (Leon-Kloosterziel et al., 2005) and rice (Nakazaki et al., 2006) PR-1 proteins identified by PlantTribes, qRT-PCR analyses have shown that the *Populus* PR-1 ortholog is strongly upregulated after infection by *M. larici-populina* (Rinaldi et al., 2007) and *Myrmicaria brunnea* (Cheng et al., 2008). The PR-2 protein has β-1,3-endoglucanase activities, which likely hydrolyze fungal cell walls, while PR-5 proteins belong to the large TLP family, which also includes osmotins and osmotin-like proteins, which are reported to act against oomycetes and fungi (Leon-Kloosterziel et al., 2005; van Loon et al., 2006). In our study, contig 51 and 52 were two unigenes that were homologous to PR-1 of *P. tomentosa*, and contig 50 and singlet 25 had a similar sequence to that of a β-1,3-glucanase gene of *P. tremula* x *P. tremuloides*. Meanwhile, the sequences of contig 49 and singlet 23 were predicted to encode TLP because of their homology to the *Nicotiana tabacum* TLP gene. Considering that several unigenes encoding PR proteins were upregulated after MLP infection, the poplar PR genes can be expected to play important roles in pathogen defense. A previous poplar study also correlated increased lignin accumulation with phenylalanine ammonia-lyase (PAL) enzyme activities after inoculation with *Entoleuca mammata*, and the results indicated that the PAL gene was often reported as marker gene of lignin deposition (Bucciarelli et al., 1998). One uni-EST in our paper, contig 47 (10 copies), was similar to the PAL gene of *P. trichocarpa*, which is indicative of a role in pathogen defense. Meyers et al. (2003) and Belkhadir et al. (2004) reported that the nucleotide-binding site and leucine-rich repeat protein resistance genes were the largest class of disease resistance genes, which play a crucial role in defending plants from a multitude of pathogens. Strikingly, a significant induction of contigs 2 and 15, which were assembled by 17 ESTs and predicted to be a *P. trichocarpa* nucleotide-biding site-type disease resistance protein, was also detected in the incompatible interaction.

Validation of rust-regulated genes using cDNA RT-PCR and qPCR

To confirm the results of the subtracted cDNA library, we randomly selected five upregulated clones (TLP, myo-inositol 1-phosphate synthase, PR-1, β-1,3-glucanase, and PAL) to analyze their expression patterns by RT-PCR during a time-course infection of *P. szechuanica* leaves at 12, 24, 48, 72, 96, and 120 hpi and mock-inoculated leaves (0 h), with ubiquitin as a positive control. The expression levels of all five clones from the subtracted cDNA library increased compared with those of the mock-inoculated leaves (0 h). The expression of β-1,3-glucanase was slightly detectable at 0 h, and myo-inositol 1-phosphate synthase was expressed in both mock and post-inoculation samples. Obviously, the expression level of the TLP gene was highest at 72 hpi, and that of PR-1 was highest at 48 hpi (Figure 2). qPCR was also performed, and the results showed that expression level of the TLP gene was increased at the latter stage of the infection process, especially at 72 hpi. Compared with mock-inoculated leaves, PR-1 gene expression was upregulated greater than approximately 70- and 35-fold at 48 and 96 hpi (Figure 3). In contrast, qRT-PCR analyses showed that *Populus* TLP and PR-1 orthologs were strongly expressed after infection by *M. larici-populina* and *M. brunnea* at 48 hpi (Rinaldi et al., 2007; Cheng et al., 2008). The differences observed between our study and previous studies may have been because of differences in study design or differences in samples.
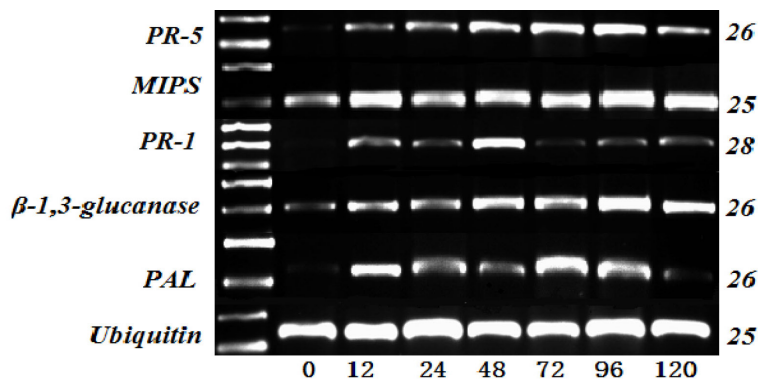
Figure 2. Suppression subtractive hybridization (SSH) results by RT-PCR during time-course infection of *Populus szechuanica* leaves at 12, 24, 48, 72, 96 and 120 h post-inoculation and non-inoculated leaves (0 h). Numbers of PCR cycles are listed on the right side. PR-5 = thaumatin-like protein gene; MIPS = myo-inositol 1-phosphate synthase gene; PR-1 = pathogenesis-related protein 1 gene; β-1,3-glucanase = beta-1,3-glucanase; PAL = phenylalanine ammonia lyase; Ubiquitin = endogenous control.
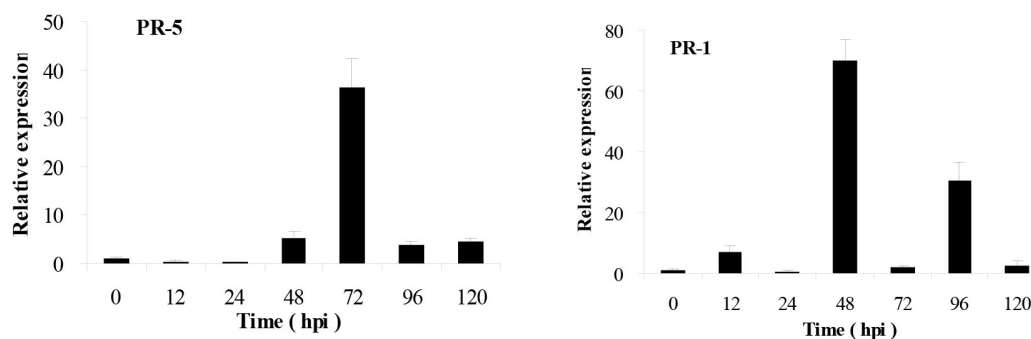
Figure 3. qPCR assay for the transcription levels of PR-5 and PR-1 during time-course infection of *Populus szechuanica* leaves at 12, 24, 48, 72, 96 and 120 hours pot-inoculation (hpi) and non inoculated leaves (0 h). PR-5 = thaumatin-like protein gene; PR-1 = pathogenesis-related protein 1 gene; Ubiquitin = endogenous control.

In conclusion, we identified 141 differentially expressed sequences enriched in post-inoculation poplar leaves using the SSH method. Based on GO annotation, 61 identified unigenes were organized into three categories representing molecular functions, biological processes, and cellular components, and five uniESTs were analyzed by RT-PCR and qPCR. The results showed that expression level of the TLP gene was highest at 72 hpi, and that of PR-1 was highest at 48 hpi, suggesting that at least the genes selected in this study may play important roles in pathogenesis.

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Supplementary material

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